Phototoxic Erythema Following PUVA Treatment: Independence of Complement

Astrid D. Rauterberg, M.D., Ernst G. Jung, M.D., Reinhard Burger, Ph.D., and Ernst W. Rauterberg, M.D.
Department of Dermatology, Mannheim (ADR, EGJ) and Institute of Immunology, Department of Clinical and Experimental Immunopathology (RB, EWR) University of Heidelberg, F.R.G.

The effect of PUVA treatment on normal human serum (NHS), on isolated PMN, or on C3-deficient guinea pigs and congenic (C3-competent) control animals was tested. At a concentration of 0.1 or 1 mM/l 8-MOP and UVA doses of 5–30 J/cm², PUVA failed to induce any detectable C3-cleavage in NHS. Furthermore, when the complement (C) activation in NHS had been induced before or after PUVA treatment by various methods, PUVA did not modulate the extent of C3-cleavage. PUVA did not affect the viability of isolated PMN, nor did it induce a release of LDH or elastase. No differences between C3-deficient and C-competent guinea pig skin exposed to PUVA were observed in erythema or histologic responses. Immunohistologic examination of specimens from normal guinea pigs revealed C3b and C3d deposits on necrotic keratinocytes, findings restricted to the PUVA-treated areas. Necrosis of keratinocytes was present in skin specimens of C3-deficient animals from PUVA-treated sites to a similar extent. However, deposits of C3-related antigens were completely absent there. From these observations, we suggest that the induction of phototoxic erythema following PUVA treatment is independent of complement.
J Invest Dermatol 94:144–149, 1990

Treatment with 8-methoxypsoralen (8-MOP) and subsequent exposure to UVA irradiation, i.e., PUVA, is widely and effectively used in therapy of psoriasis, lichen ruber, mycosis fungoides, and other dermatoses. One of the most frequent side effects is a delayed erythema, which represents an interesting tool for studies of mechanisms of cutaneous inflammation in vivo.

Acute phototoxicity due to psoralen and UVA has several characteristics which distinguish it from cutaneous responses to UVB-radiation (sunburn). Erythema caused by PUVA treatment has a longer latent period between irradiation and onset, with its maximum at 48–72 h. The mechanism leading to the development of PUVA phototoxicity and the mediators which are liberated have not yet been identified [1]. Cyclooxygenase inhibitors like indomethacin have been shown to inhibit UVB-erythema. However, they are ineffective in modulating PUVA-induced erythema. Therefore, it is probable that substances other than products of the cyclooxygenase pathway, i.e., prostanoids, are acting as mediators of this type of inflammation.

The complement system is one of the most important effector systems of inflammation. The fact that, in the past, numerous phototoxic substances have been shown to promote their phototoxic effects by a photoactivation of the complement system led us to hypothesize that complement activation may be a common causative factor in the pathogenesis of phototoxic cutaneous responses. Our hypothesis was that PUVA phototoxicity might also be mediated by activated complement components. This concept was supported by the earlier observation of complement activation after PUVA in suction blister fluid of the irradiated skin [2].

In the following experiment, the effects of PUVA on the complement system in normal human serum and on polymorphnuclear leukocytes (PMN) were analyzed. Furthermore, the development of PUVA-induced phototoxicity was studied in normal and C3-deficient guinea pigs. Lesional skin of the guinea pigs was examined immunohistologically for evidence of C-activation.

MATERIALS AND METHODS

Animals The studies were performed on outbred Hartley strain albino female guinea pigs ranging in weight from 350 to 500 g and possessing an unimpaired complement system. In parallel experiments, white inbred guinea pigs genetically deficient in C3 were used [3]. Congenic guinea pigs from the parental C3-competent

Manuscript received January 19,1989; accepted for publication August 10, 1989.
A preliminary report of this work was presented at the "16. Tagung der Arbeitsgemeinschaft Dermatologische Forschung," November 26, 1988, in Köln, FRG.
Dr. Rauterberg was the recipient of a grant (No 160/1–1) from the Deutsche Forschungsgemeinschaft, Bonn, FRG.
Dr. Burger's present address is the Robert-Koch Institute, Bundesgesundheitsamt, Berlin, FRG.
Dr. Rauterberg's present address is the Deutsche Klinik für Diagnostik, Wiesbaden, FRG.
Reprint requests to: Dr. Astrid D. Rauterberg, Hautklinik des Klinikum Mannheim der Universität Heidelberg, P.O. Box 10 00 23, D-6800 Mannheim, FRG.

Abbreviations:
8-MOP: 8-methoxypsoralen
ABC: avidin-biotin-complex
APAAP: alkaline phosphatase/anti-alkaline phosphatase
C: complement
DMSO: dimethylsulfoxide
EDTA: ethylenediaminetetraacetic acid
ELISA: enzyme-linked immunosorbent assay
FITC: fluoresceinisothiocyanat
HBSA: Hank's balanced salt solution with bovine serum albumin without calcium
IF: immunofluorescence
LDH: lactic dehydrogenase
PBS: phosphate-buffered saline
PMN: polymorphonuclear leukocytes
PUVA: 8-methoxypsoralen and ultraviolet A radiation
RIA: radioimmunoassay
UVA: ultraviolet A
UVB: ultraviolet B
UVC: ultraviolet C
XIE: crossed immunoelectrophoresis

0022-202X/90/$03.50

inbred strain served as controls. All animals had free access to water and standard laboratory food.

Light Source The animals received irradiation from a PUVA 4000 lamp (Waldmann, Villingen-Schwenningen, FRG), which has an emission spectrum of 320–390 nm with a maximum at 365 nm. The output at wavelengths shorter than 320 nm was blocked by UVB and UVC filters. The distance from the light source to the target was 25 cm. UV light irradiance was measured with a thermopyle (Kipp and Zonen, Delft, Holland).

PUVA Treatment At the dorsal surface of each animal an area of 6 × 6 cm was shaved with electric shears. We minimized the possible effect of irritation from shaving by performing PUVA treatments 24 h after shaving. The dorsal surface was divided into four areas, each measuring 3 × 3 cm. One area was used for each of the following treatments: PUVA, UVA only, 8-MOP only, and untreated control.

A 0.15% solution of 8-MOP (Meladinine, Basotherm, Biberach, FRG) was applied topically on the right side (6 × 3 cm) of the shaved back (20 μl/cm² skin surface) 45 min before UVA. The left side remained without 8-MOP treatment. The caudal half of the shaved area was covered with light occlusive tape. The cranial part was irradiated with UVA in total single doses ranging from 1 to 10 J/cm². Both C3-deficient animals and C3-competent controls received a single dose of 5 J/cm² UVA, a dose which was erythrogenic in normal outbred guinea pigs.

At 1, 24, 48 and 72 h after UVA exposure, erythema was graded on a semiquantitative scale from 0 to ++++. Punch biopsy specimens (5 mm diameter) were taken from each area 1, 2, 3, 5, 7, and up to 12 d after treatment. The specimens were snap-frozen in melting isopentan, cooled by liquid nitrogen, and stored at −80°C until they were used for immunohistologic examination.

Immunohistologic Techniques 5 μm thick cryostat sections cut at −20°C were placed on microscope slides, air dried, and fixed in acetone. APAAP staining was performed according to the method of Cordell [4]. Briefly, sections were incubated overnight with monoclonal antibodies to guinea pig C3 described previously [5]. Ascites diluted 1:80 or 1:400 was used as the source of antibody. The sections were rinsed in PBS, pH 7.4, then incubated with the bridging antibody (rabbit anti-mouse IgG 1:25, Dakopatts, Copenhagen, Denmark) for 25 min. After being washed again in PBS, sections were incubated with the APAAP complexes (Dakopatts, 1:10 diluted). Alkaline phosphatase was visualized by fast red method.

Biotin-streptavidin-peroxidase staining was performed as previously described [6], with polyclonal rabbit antibodies to human C3c, C3d, and C4 which had been shown to react predominantly with tissue bound C3b, C3d-g/C3d, or C4d, respectively (Dakopatts, diluted 1:400; the specificity towards guinea pig antigens was confirmed by dotblot analysis, Ouchterlony, or ELISA). After incubation for 30 min, sections were washed in PBS, incubated with a biotin goat anti-rabbit antibody (1:400 diluted; Dianova, Hamburg, FRG), again rinsed in PBS, then incubated with streptavidin-peroxidase (1:400; Amersham-Buchler, Braunschweig, FRG), and finally stained with 0.5 mg/ml diaminobenzidine (Fluka, Buchs, Switzerland) and 0.012% H_2O_2. Binding of the rabbit anti-C3d and -C3c antibodies was visualized, in parallel, by the ABC-technique (Camon/Vector, Wiesbaden, FRG). In these sections, antibody unrelated biotin binding sites of the tissue had been blocked by a biotin blocking kit (Camon/Vector, Wiesbaden, FRG).

Additionally, a portion of each biopsy specimen was stained with hematoxylin eosin and periodic-acid-Schiff. Sections were mounted in buffered glycerol and examined under light microscope (Zeiss, Frankfurt, FRG). Photomicrographs were taken on a Kodak Ektachrome, 50 ASA, at a final magnification of ×100.

In Vitro Irradiation of Human Serum and Complement Assays 8-MOP pure substance (Basotherm, Biberach, FRG) was added to normal human serum at a final concentration of 0.1 or 1 mM/l, respectively. The test tubes were wrapped in tin foil to minimize the effect of ambient ultraviolet irradiation, and cooled on ice.

60 min later, sera were transferred to petri dishes, placed on ice at a distance of 25 cm from the light source, and irradiated in doses from 5 to 30 J/cm². To evaluate the effect of UVA only, normal human serum was irradiated in parallel in the absence of 8-MOP. Other aliquots containing 8-MOP were not exposed to UVA. Negative controls received neither 8-MOP nor UVA. Complement activation of the positive controls was induced by addition of 25 mg/ml zymosan to normal human serum and incubation for 60 min at 37°C.

After irradiation, samples were incubated in darkness at 4°C or 37°C, respectively. At different time intervals, i.e., 1, 2, 4, 24, and 48 h after irradiation, aliquots were taken from each sample. To each aliquot a stock solution (0.1 M/l Na_2 EDTA, pH 7.2) was added to give a final concentration of 0.01 M/l EDTA to stop further C activation. Aliquots were snap-frozen in liquid nitrogen and stored at −80°C.

Normal human serum from the same healthy donor as above was treated as mentioned with PUVA, 8-MOP, or UVA before or after C-activation by four different methods [7]: incubation for 120 h at 37°C, or the addition of zymosan, $MgCl_2$, or aggregated IgG, respectively. In the untreated controls, each mode of activation resulted in a defined cleavage of C3 (40%–80%, respectively) as shown by two-dimensional crossed immunoelectrophoresis (XIE).

XIE was performed according to Laurell [8] with modifications suggested by Weeke [9] in 1% agarose on 10 × 10-cm glass slides. Rabbit antiserum to human C3c and C3d was purchased from Dakopatts and was present at concentration of 0.75 and 1.5 μl/cm², respectively, in the gel during the second dimension.

For C3a measurements, a commercially available RIA test kit was used (Amersham-Buchler, Braunschweig, FRG) applying the above-mentioned zymosan activated serum as positive control.

In Vitro Irradiation of PMN PMN were obtained from venous blood of healthy adult donors by the method described by Haslett, Guthrie, Kopaniak et al [10]. Briefly, PMN were isolated by a plasma-percoll gradient technique, washed in platelet poor plasma and HBSA, and adjusted to 2–5 × 10^6 cells/ml. This final preparation contained 90–95% neutrophils. Viability was higher than 95% as assessed by trypan blue exclusion assay.

8-MOP was diluted in DMSO to give a final concentration of 1 or 10 μg/ml in the culture medium, i.e., 4.63 μM/l or 46.26 μM/l, respectively. Final concentration of DMSO never exceeded 0.1% (v/v). After incubation in darkness for 60 min at room temperature, cells were transferred to petri dishes and irradiated with UVA in doses from 3 to 5 J/cm². Other samples were only exposed to UVA or to 8-MOP, respectively. Controls remained untreated. After irradiation, exposure of the cells to UVA was kept to a minimum.

The trypan blue dye exclusion test of viability was performed first 1 h after PUVA treatment and again after an additional incubation period of 24 h at 37°C.

A part of the cells was centrifuged after PUVA, resuspended in normal human serum 1:2 diluted by HBSA, and incubated for 15 min at 37°C. Supernatants of these cells were examined for evidence of C3-activation by a C3a RIA (Amersham). The cells and the pellets were washed in HBSA and stained by conventional immunofluorescence technique with FITC-labeled polyclonal antibodies to C3c (Dakopatts, Copenhagen, Denmark, in a dilution of 1:30 with PBS). Lactic dehydrogenase in the supernatants was measured with sodium pyruvate as substrate. Elastase release was assayed according to the method of Bieth, Spiess, and Wermuth [11] with succinylnitroanilid as substrate (Sigma, Deisenhofen, FRG). Supernatants of cells lysed by TritonX100 served as positive control for both LDH and elastase assay. Specific release was expressed as calculated according to the following formula:

$$X_1 = \frac{\text{release in experiments} - \text{background release}}{\text{total positive control} - \text{background release}}.$$

RESULTS

In Vitro Irradiation of Serum To investigate whether PUVA could induce a direct activation of serum complement, we analyzed its effect on C3 by XIE, varying both UVA dosage and 8-MOP concentration. A representative result of several experiments is shown in Fig 1. There were no significant differences in immunoelectrophoretical C3-conversion between PUVA, UVA only-, and 8-MOP only-treated, and untreated sera. XIE of all samples incubated at 37°C for various times showed additional extra-anodal precipitation peaks representing cleavage products of C3, i.e., C3b, C3c, and C3d/C3d-g. No C-activation occurred in samples incubated at 4°C. Addition of 8-MOP and/or exposure to UVA alone did not alter these effects. The positive control (zymosan) revealed an 80 ± 2% (mean ± SD) cleavage of C3.

C activation induced in normal human serum at 37°C by zymosan, IgG-aggregates, $MgCl_2$, or prolonged incubation was, furthermore, not modulated by the addition of 8-MOP or UVA irradiation or by PUVA before treatment or thereafter (data not shown).

Sera were examined in parallel for evidence of C activation by C3a-RIA. The C3a concentrations of all sera were in the normal range (Table I). Addition of 8-MOP and/or UVA irradiation did not enhance C3a generation in serum or EDTA-plasma. Positive control (zymosan) showed about 82 ± 3% of maximal C3a generation.

In Vitro Irradiation of PMN Assessment of leukocyte viability by means of trypan blue exclusion revealed that cell viability was not affected even at high concentrations of 8-MOP (10 ng/ml) and/or high UVA dosage (5 J/cm²). Viability 1 h after irradiation was between 95% and 100% in all samples (Table II). After an incubation period of 24 h at 37°C, the ability of 8-MOP and/or UVA-treated leukocytes to exclude the dye was only slightly diminished, i.e., neither PUVA, UVA, nor 8-MOP had any direct or prolonged effect on PMN viability. The extent of LDH release was similar in all four tested groups.

Elastase release of PMN was not notably affected as well by PUVA, UVA, or 8-MOP, as compared to the untreated controls. Supernatants of cells which had been incubated with NHS after PUVA treatment showed no significant C3a generation and did not differ in this respect from the other three groups (8-MOP, UVA, or untreated cells). Direct immunofluorescence did not reveal any C3b or C3d deposition on the surface of the PMN in either groups.

Table I. C3a Release in NHS After PUVA Treatment

	C3a (ng/ml)
NHS + 8-MOP[a] + UVA[b]	432
NHS + 8-MOP[a]	504
NHS + UVA[b]	456
NHS untreated	468
Negative controls	
Plasma[c] + 8-MOP[a] + UVA[b]	218
Plasma[c] + 8-MOP[a]	222
Plasma[c] + UVA[b]	216
Plasma[c] untreated	222

[a] 8-MOP added 45 min prior to UVA at final concentration of 1×10^{-3} M/l.
[b] Samples irradiated with 30 J/cm² UVA.
[c] Plasma containing 10 mM/1 EDTA.

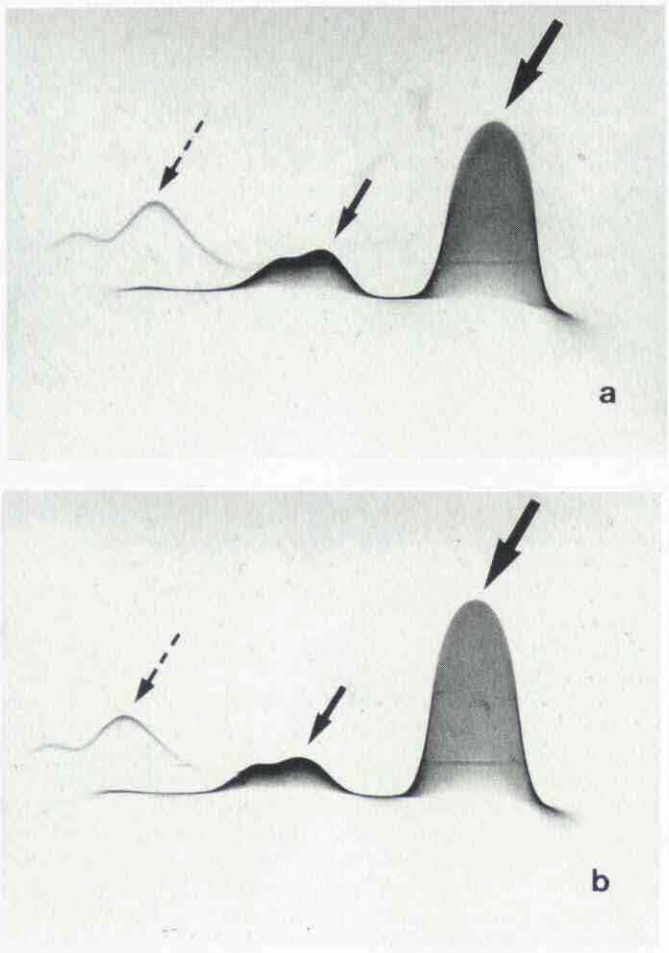

Figure 1. Crossed immunoelectrophoresis of (*a*) PUVA-treated (1 mM 8-MOP; 5 J/cm² UVA) and (*b*) untreated normal human serum. Anode left. *Arrows* point to the positions of uncleaved C3 *(big arrow)*, C3b *(little arrow)*, and C3d *(arrow with dotted line)*. No differences in C3 conversion were observed.

In Vivo Studies PUVA treatment of normal guinea pigs resulted in the development of erythema 24 h after irradiation with a maximum at 48 h (Fig 2). Clinical changes were associated with acanthosis and the appearance of necrotic keratinocytes (sunburn cells) between 2 and 5 d after PUVA as shown by histologic examination (Fig 3*a'*). Immunohistologic staining consistently demonstrated deposits of C3-related antigens (Fig 3*a*) and C4d (not shown) on necrotic keratinocytes. C3d/C3d-g was similarly present at those sites. C3-related antigen deposition could be visualized by both techniques, i.e., by the polyclonal antibodies and the ABC technique and by monoclonal antibodies against C3 and the APAAP method.

At sites only treated either with UVA (Fig 2*b*) or 8-MOP (Fig 2*c*), respectively, no visible changes, such as erythema, were noted. Histologic and immunohistologic examination of these areas showed normal epidermis and dermis (Fig 3*c*, *c'*).

Similar responses to PUVA were elicited in inbred guinea pigs congenitally deficient in C3 and in control animals from the congenic C3-competent inbred strain. Delayed erythema was identical in intensity and time course. Histologic examination revealed similar findings as in the outbred animals: acanthosis, blistering, and necrotic keratinocytes were demonstrable between the second and the fifth day in both groups of animals.

Immunohistologic findings in PUVA-treated skin, however, were quite different in C3-deficient guinea pigs. As shown in Fig 3, deposits of C3-related antigens (Fig 3*b*) were completely absent, while the extent of acanthosis and necrosis of keratinocytes (Fig 3*b'*) was similar to that found in the PUVA-treated areas of C3-competent animals.

DISCUSSION

The results of the present study show that the cutaneous phototoxic reaction following PUVA treatment develops independent of the complement system. We analyzed the question of a possible involvement of complement by four independent approaches: (1) the direct effect of PUVA on C3 conversion in fresh human serum; (2) the modulating effects of PUVA on various types of ongoing C

Table II. Viability and Elastase and LDH-Release of PMN's Following PUVA-Treatment

Group	8-MOP 10 μg/ml	UVA 5 J/cm²	Viability (%) 1 h	Viability (%) 24 h	LDH (% spec. release) 1 h	Elastase (% spec. release) 1 h	Elastase (% spec. release) 24 h
I	+	+	99	86	0	0	1
II	−	+	95	86	16	0	3
III	+	−	99	84	0	11	4
IV	−	−	98	92	0	1	10

activation in vitro; (3) the indirect effects of PUVA on serum complement via activation or damage of PMN, and (4) the development of PUVA-induced erythema and immunohistologic alterations of the skin in C3-deficient and C3-competent guinea pigs.

Measurement of C3 cleavage or its modulation was assayed by crossed immunoelectrophoresis and C3a-RIA. In related studies of UV exposure, C3-cleavage products were detected by XIE in normal human serum containing demethyltetracycline [12], chlorothiazide [13] or chlorpromazine [14], thus indicating C activation. UV irradiation of sera from patients with erythropoetic porphyria and porphyria cutanea tarda, as well as local in vivo UV irradiation of patients with porphyrias, resulted in C3 conversion in XIE [13,15]. XIE would have been sensitive enough to detect as little as 5% conversion of serum C3 in our hands (interassay variance < 4%; data not shown). Our observations were corroborated by the highly sensitive C3a-RIA as a second test method to confirm C3 cleavage.

However, in our measurements there was no evidence for C activation in PUVA-treated serum as compared to UVA-treated, 8-MOP-treated and untreated serum even at high 8-MOP concentrations and over a wide range of UVA dosages. To investigate whether PUVA modulates an ongoing C activation in vitro, we induced complement activation before and after PUVA and measured C3 cleavage by XIE. PUVA did not result in potentiation or inhibition of C3 cleavage irrespective of the type of C activation.

Since a direct serum-C activation in a cell-free environment was ruled out, we analyzed the PUVA-induced release of C3-cleaving enzymes from PMN. The extent of PMN activation in vitro is correlated with the amount of granulocyte-elastase released into the supernatant [16]. The known potency of granulocyte-derived elastase to cleave and activate C3 suggests that liberation of this enzyme is the cause of antibody-independent C3 activation in vivo, which is observed in various types of inflammation [17]. LDH concentration in the supernatant and the dye exclusion tests are good parameters

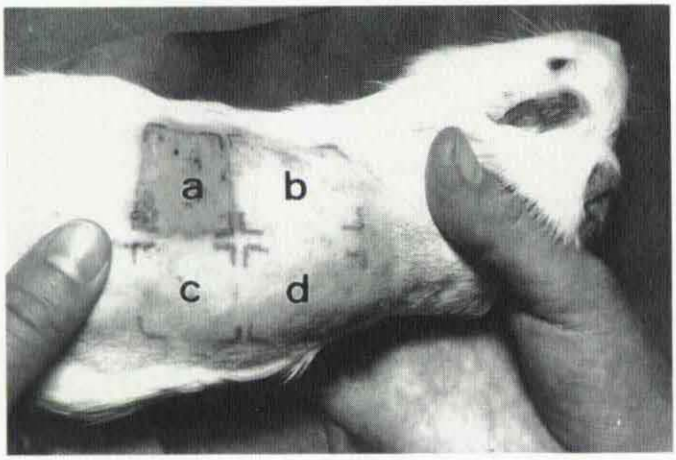

Figure 2. Erythema 72 h after local treatment with (*a*) PUVA (8-MOP topically; 5 J/cm² UVA), (*b*) UVA (similar dose), or (*c*) 8-MOP. (*d*) Untreated control. An erythema was elicited exclusively in and limited to the PUVA-treated area.

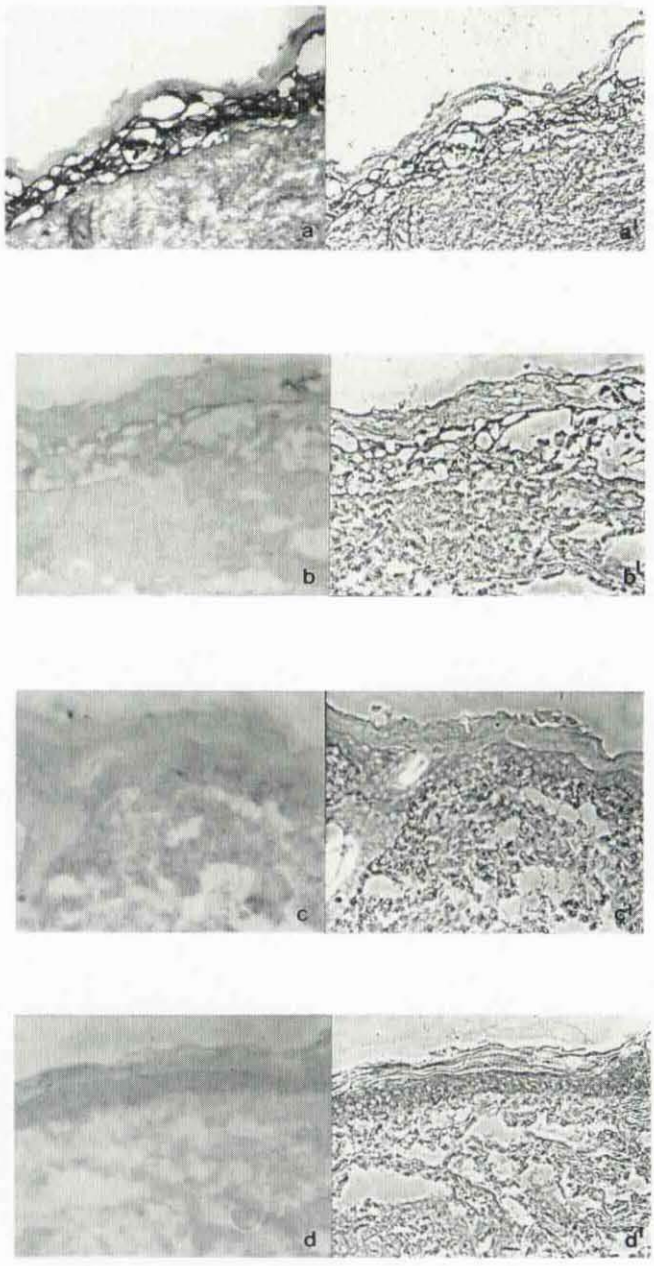

Figure 3. Paired photomicrographs (*left side:* immunohistologic staining; *right side:* phase contrast image taken in parallel) of guinea pig skin specimens after staining for tissue bound C3-related antigens (ABC peroxidase technique, rabbit anti-C3c). (*a*) PUVA-treated (8-MOP topically; 5 J/cm² UVA) normal guinea pigs revealed remarkable deposits of C3-related antigens on necrotic keratinocytes. Note the acanthosis and extensive formation of intraepidermal small blisters (*a'*). C3-related antigens were absent (*b*) in the similarly damaged epidermis (*b'*) of the C3-deficient animals. In UVA-treated gp skin no immunohistologic changes were noted, neither in normal (*c/c'*) nor in C3-deficient gp (*d/d'*). Magnification of all micrographs, ×120.

for measuring damage and/or viability of PMN, respectively [18]. However, in our study neither LDH nor elastase from PMN nor PMN viability was affected by PUVA.

We had focused our attention on viability and on deposition of C3 on PMN because damaged or necrotic cells are capable of activation of the complement system via the alternative pathway [19]. Our experiments with PUVA-treated PMN which were subsequently incubated with serum for detection of such delayed C activation were completely negative. Similarly, the C3a-assay failed to detect any C3-cleavage under those conditions. Our negative findings are mostly consistent with recent observations of other investigators in which PUVA did not remarkably affect the viability or biologic functions of PMN in vitro and in vivo [20–24].

The most convincing evidence for a complement independence of PUVA phototoxicity was obtained in our study by PUVA treatment of guinea pigs congenitally deficient in C3 [3]. Those animals developed phototoxic dermatitis after PUVA identical in time course and intensity to that of normal congenic C3 competent control animals.

These findings contrast with the skin reactions occurring with other exogenous or endogenous phototoxic reagents in decomplemented animals. Phototoxic reactions induced by demethyltetracycline, hematoporphyrin, and chlorpromazin were markedly suppressed in cobra venom factor decomplemented guinea pigs as compared to control animals, indicating that an intact complement system is required for the full development of these types of phototoxic reactions [12,14,25].

In our experiments, histologic changes after PUVA, i.e., acanthosis, necrosis of keratinocytes were identical in C3-deficient and normal guinea pigs. However, deposits of C3 metabolites (predominantly C3d) as demonstrated by immunohistology were exclusively on necrotic keratinocytes of C3-competent guinea pigs. The presence of a fully developed PUVA erythema and its typical pathohistologic features in the absence of C3 deposits (in the C3-deficient animals) is consistent with C3 activation and deposition of C3d in normal guinea pigs occurring primarily as an epiphenomenon. These changes might be induced by the necrosis of keratinocytes or could be the result of a PUVA-induced alteration to glycoproteins on keratinocyte surface membranes [26].

The absence of immunoglobulin or complement "C3" deposits in the skin of PUVA-treated patients has also been reported by other laboratories [27,28]. We had the chance to examine one skin biopsy taken from a psoriatic patient after development of a PUVA erythema. We failed to detect any indication of local C activation when we stained the skin biopsy for C1q, C4b, C4d, C3b, C3d, factor H, factor P, C5, C9, and C5b-9-neoantigens using both direct immunofluorescence or monoclonal antibodies in the APAAP method (data not shown). Recently "C3" deposition at the dermo-epidermal junction and around the upper dermal vessel was reported by others in seven patients developing blisters after PUVA [29]. The authors suggested it was unlikely that C activation had mediated the epidermal damage because no accumulation of neutrophils accompanied C deposition. We have examined a biopsy specimen taken from a blister occurring 12 h after local PUVA therapy in a patient with an eczema and an increased sensitivity to UVA irradiation. Immunohistologic examination demonstrated remarkable IgM and complement deposits (predominantly C4d, C3d, C3b, factor H, and C5b-9 complexes) on the necrotic keratinocytes and in the upper dermal vessels (Fig 4). These findings confirm that complement activation and immunoglobulin deposition, which are absent in PUVA erythema, can occur in association with more severe phototoxic tissue damage, such as blister formation.

The pathogenic role of complement in PUVA blister formation needs to be clarified by future studies. Complement activation seems not to be essential for the development of the PUVA-induced delayed erythema. Further studies will have to elucidate whether other potent mediators (such as eicosanoids, platelet activating factor, metabolites of keratinocytes, or cytokines) are involved in erythema formation after PUVA treatment.

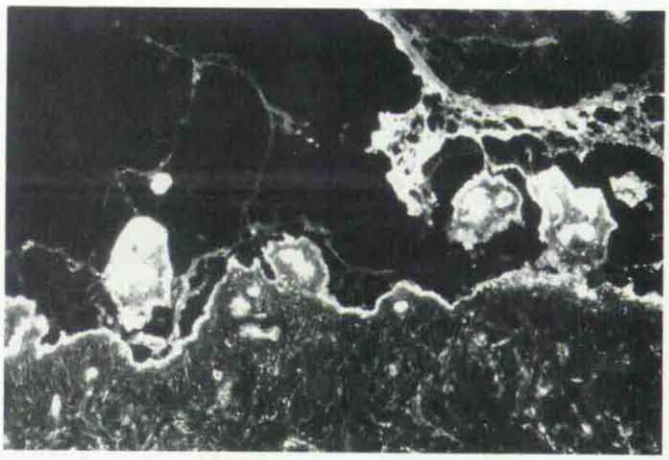

Figure 4. Direct immunofluorescence staining of a biopsy specimen from a blister occurring 12 h after local PUVA (8-MOP topically; 0.5 J/cm² UVA) in a patient with an eczema. Heavy C3d deposits were present on necrotic keratinocytes in the blister roof and bottom and around superficial dermal vessels. Magnification, ×100.

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